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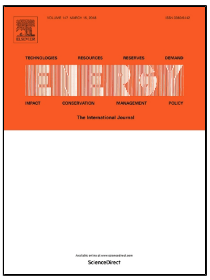

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Assessment of continuous fermentative hydrogen and methane co-production using macro- and micro-algae with increasing organic loading rate

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Abstract

A two-stage continuous fermentative hydrogen and methane co-production using macro-algae (*Laminaria digitata*) and micro-algae (*Arthrospira platensis*) at a C/N ratio of 20 was established. The hydraulic retention time (HRT) of first-stage H_2 reactor was 4 days. The highest specific hydrogen yield of 55.3 mL/g volatile solids (VS) was obtained at an organic loading rate (OLR) of 6.0 gVS/L/d. In the second-stage CH_4 reactor at a short HRT of 12 days, a specific methane yield of 245.0 mL/gVS was achieved at a corresponding OLR of 2.0 gVS/L/d. At these loading rates, the two-stage continuous system offered process stability and effected an energy yield of 9.4 kJ/gVS, equivalent to 77.7% of that in an idealised batch system. However, further increases in OLR led to reduced hydrogen and methane yields in both reactors. The process was compared to a one-stage anaerobic co-digestion of algal mixtures at an HRT of 16 days. A remarkably high saline level of 13.3 g/L was recorded and volatile fatty acid accumulation were encountered in the one-stage CH_4 reactor. The two-stage system offered better performances in both energy return and process stability. The gross energy potential of the advanced gaseous biofuels from this algal mixture may reach 213 GJ/ha/yr.

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30 **Keywords:** Macro-algae; micro-algae; two-stage co-fermentation; hydrogen; methane

1. Introduction

In recent years there is an increased interest in producing advanced biofuels from alternative feedstocks. The need to improve energy yields and allay sustainability concerns including land use change of first and second generation biofuels have led to research of algae (both macro and micro) as viable substrates for the production of advanced biofuels. Algal biofuels can overcome the food-or-fuel debate associated with first generation biofuels [1, 2] and do not face the complex conversion processes required for second generation biofuel production [3, 4]. Aquatic algae possess several advantages over terrestrial plants. Firstly, both macro-algae and micro-algae have higher growth rates and biomass productivities as compared to agricultural crops [5-7]. Secondly, the cultivation of algae may not require arable lands or fresh water. A win-win situation can be achieved through coupling algae production with wastewater treatment [8-10]. Thirdly, algae may provide continuous biomass supply throughout the year with optimised cultivation such as CO_2 supplementation using flue gas for micro-algae [11, 12] and efficient preservation such as ensiling for macro-algae [13].

Production of liquid biofuels (such as biodiesel and bioethanol) using algae biomass has been extensively explored [14, 15]. However, the parasitic energy demand for the generation of liquid biofuels from raw feedstocks exceeds that in the conversion from substrates to gaseous biofuels such as biohydrogen and biomethane [16-18], leading to comparatively lower overall energy efficiencies. Besides, gaseous biofuels offer more utilisation options, including: compression for vehicles fuels; injection into the existing natural gas grids for use as renewable heat in industry such

as breweries [19]; on site electricity generation using internal combustion engines [20]; or increased efficiency through use of biomethane from the gas grid at combined cycle gas turbines.

Biological hydrogen production through dark hydrogen fermentation of algae biomass shows advantages over conventional energy-intensive hydrogen-producing methods such as steam methane reforming [21] due to the mild reaction conditions and renewability of the produced hydrogen [22]. However, limited energy conversion restricts its application. An alternative gaseous product biomethane generated through biological anaerobic digestion of algae biomass with better energy output has been analysed in previous studies [15, 23, 24]. Nevertheless, some major bottlenecks still restrict the application of this process. The abundant recalcitrant organics such as polyphenols in macro-algae [5] and triglycerides in micro-algae are not readily digested by the microbes and thereby decrease the biodegradability of biomass [23]. In addition, the rigid cell wall structures of algae act as barriers between the intracellular biodegradable contents and anaerobic microbes, hence hindering the degradation and methanogenesis of algae biomass in anaerobic digestion process [24]. To tackle this problem, a two-stage process combining hydrogen fermentation and anaerobic digestion can serve as a promising solution. The two-stage set-up separates the process phases and optimises the operational conditions for each. In the first stage of hydrogen fermentation, the anaerobic fermentative bacteria (AFB) favour the pH condition of 5-6 where they can efficiently degrade the large-molecular-weight organics such as carbohydrates and proteins into gaseous hydrogen, carbon dioxide,

and liquid soluble metabolic products (such as volatile fatty acids (VFAs), alcohols, and lactic acid) in a short retention time (2-4 days) [22]. Subsequently, the liquid fermentation effluents rich in small-molecular-weight VFAs and alcohols can be readily utilised by the methanogenic organisms in the second stage of anaerobic digestion. Therefore, compared with one-stage anaerobic digestion, the two-stage process presents better energy yields with improved biogas production and significantly shortens the overall retention time with concurrent increase in organic loading rates (OLRs). Yang et al. [25] used lipid-extracted residues of microalgae *Scenedesmus* for two-stage batch fermentative hydrogen and methane co-production and obtained a 22% increase in methane yield and a 27% increase in energy efficiency in contrast to that in one-stage anaerobic digestion. Massanet-Nicolau et al. [26] investigated the two-stage continuous fermentative hydrogen and methane co-production of pelletized grass, which exhibited an overall energy yield of 11.74 kJ/g volatile solids (VS) with an increase of 13.4% compared with one-stage anaerobic digestion. Process stability was maintained whilst the hydraulic retention time (HRT) was greatly shortened from 20 days in the one-stage to 12 days in the two-stage process [26].

Apart from relatively limited biodegradability of algae compared with some first generation feedstocks [5], the intrinsic compositional unbalance of certain algae biomass (in particular micro-algae biomass) can impair the anaerobic digestion process [27]. Proteins occupy a large portion of organics in micro-algae, leading to a low C/N ratio in the biomass. The excessive nitrogen is released in the form of

ammonia during the degradation of proteins, resulting in severe decrease in the microbial activities of methanogenic microbes [28]. By contrast, some species of macro-algae, such as brown seaweeds *Laminaria digitata* and *Saccharina latissima*, contain rich carbohydrates and have a high C/N ratio when harvested at optimum times [5]. This can in certain cases lead to limited nitrogen supply for the basic metabolisms of AFB in hydrogen fermentation and the methanogens in anaerobic digestion [29]. The optimum C/N ratio was suggested to be 20-30 for algal feedstocks [21, 30]. Thus, adjusting the C/N ratio by mixing nitrogen-rich micro-algae and carbon-rich macro-algae as co-substrates offers an excellent strategy to improve the process performances of both hydrogen fermentation and anaerobic digestion. Xia et al. [29] mixed micro-algae *Arthrospira platensis* and macro-algae *L. digitata* for batch fermentative hydrogen production and achieved an optimal H_2 yield of 85.0 mL/gVS at a C/N ratio of 26.2. A study on the continuous one-stage anaerobic digestion of mixed *A. platensis* and *L. digitata* at a C/N ratio of 25 was conducted and the highest specific methane yield (SMY) of 273.9 mL/gVS was recorded at an OLR of 3.0 gVS/L/d and an HRT of 28 days [27]. Although many micro-algae species thrive in tropical and sub-tropical waters while macro-algae are commonly found in temperate sea, the micro-algae cultivation in temperate regions using seawater and flue gas from coal-fired power plants provides the possibility of harvesting micro- and macro-algae biomass in the same place [2, 5, 12].

The authors previously conducted a two-stage batch fermentative hydrogen and methane co-production using co-substrates of macro-algae (*L. digitata*) and micro-

algae (*Chlorella pyrenoidosa* and *Nannochloropsis oceanica*) [31]. The micro-algae biomass supplied nitrogen to balance the C/N ratio of the algal mixtures. Co-fermentation facilitated the hydrolysis and acidogenesis of the algal co-substrates and further boosted the energy conversion in anaerobic digestion. Although the batch co-fermentation provided some innovative findings, these experimental configurations have significant limitations. Batch systems allow sufficient guaranteed retention times, efficient mixing and anaerobic conditions; they also allow an optimum inoculum to substrate VS ratio of 2:1 which minimises inhibitory effects such as accumulation of volatile fatty acids and ammonia. Batch assays have limited replicability compared with likely industrial applications. In the majority of commercial industrial applications, the loading of reactor is continuous. As such it is necessary to undertake continuous laboratory experiments to assess the impact of higher OLRs and shorter HRTs for a prosperous and stable fermentation process. Economics dictate the need for high processing capability and biofuel outputs for minimum size of reactor system. Therefore, continuous two-stage laboratory co-fermentation is essential to address long term optimised operational conditions. Nevertheless, to date, long term continuous two-stage co-fermentation of micro- and macro-algae biomass remains uninvestigated in literature. This paper will address this knowledge gap in the state of the art through the following objectives:

(1) Assess co-generation of hydrogen and methane using the mixture of macro-algae (*L. digitata*) and micro-algae (*A. platensis*) at the optimal C/N ratio of 20 with increasing OLRs.

(2) Evaluate the effects of different OLRs and HRTs on the specific hydrogen yields (SHYs), the acidification yields in first-stage dark hydrogen fermentation and the SMYs in second-stage anaerobic digestion.

(3) Compare the performances of two-stage and one-stage systems on the overall energy conversion and process stability.

(4) Estimate the gross energy potential of this advanced gaseous biofuel system.

2. Materials and methods

2.1 Algal biomass and inocula

The macro-algae *L. digitata* was naturally grown in the open sea and collected in September in West Cork, Ireland. The harvested *L. digitata* was washed with tap water to remove attached sands and other impurities, and then cut to small particles (4-5 mm) by a mincer (Buffalo Heavy Duty Mincer CD400). The micro-algae powder of *A. platensis* was purchased from Bluegreen Life Foundation Inc. (Lewes, DE, USA). Both macro- and micro-algal samples were cryopreserved at -20 °C before the experiment.

The hydrogen inoculum used in biohydrogen potential (BHP) test and continuous hydrogen reactor was taken from the anaerobic sludge of an Irish farm digester. The original sludge was heated at 100 °C in an autoclave (Sanyo MLS-3780, Japan) for 30 min to inactivate methanogens and subsequently acclimatized 3 times (3 days each time) using a modified culture medium to activate the spore-forming hydrogenogenic bacteria. The compositions of the modified medium were detailed in our previous

study [31].

The inoculum used in the biomethane potential (BMP) test and continuous digestion reactors was obtained from the digestate of an existing laboratory scale seaweed anaerobic digester. The methane inoculum was degassed at a temperature of 37 °C for 7 days before the experiment.

2.2 Biohydrogen and biomethane potential tests

The two-stage batch BHP and BMP tests on the mixture of *L. digitata* and *A. platensis* were conducted in triplicate in an AMPTS II system (Bioprocess Control, Sweden).

In the BHP test, 3 g VS of the algal substrate were added to each glass bottle and then the liquor volume was adjusted to 270 mL using distilled water. Subsequently, 30 mL of hydrogen inoculum was added into each bottle to make the total working volume 300 mL. The VS portions of the two algal biomass in each bottle were calculated to effect a C/N ratio of 20: 2.82 gVS of *L. digitata* mixed with 0.18 gVS of *A. platensis*. The initial pH was adjusted to 6.00 ± 0.05 with 1 M NaOH and 1 M HCl solutions. All bottles were sealed with rubber stoppers and purged with N_2 for 5 min to maintain anaerobic conditions, and then placed in a water bath at a temperature of 37 °C for 4 days. Stirrers which were set to switch between on and off for 60 s periods with a mixing speed of 60 rpm were applied to the bottles. Carbon dioxide in the produced gas was absorbed by 80 mL of 3 M NaOH solution and then the hydrogen gas flow was recorded by a gas tipping device based on water displacement. The

recorded hydrogen gas volumes were automatically normalised to standard temperature and pressure (STP) and zero moisture content by the AMPST II system.

After the BHP test, the effluent in each bottle was analysed and then prepared for subsequent BMP test. The pH values of effluents were adjusted to 8.00 ± 0.05 with 1 M NaOH and then inoculated with methane inoculum at the inoculum to substrate VS ratio of 2:1. The total working volume of each bottle was 400 mL and the BMP test ran for 26 days so that the two-stage batch BHP and BMP tests duration reached 30 days. All the other BMP test settings were the same as those in the BHP test. A control group with just blank inocula (no substrates) was established and all the hydrogen and methane volumes produced from experimental groups were corrected for the ones produced from control group.

2.3 Set-up and operation of continuous reactors

Four lab-scale (5 L) continuously stirred tank reactors (CSTR), which comprised of one H_2 reactor and three CH_4 reactors, were used for the continuous fermentation trials as shown in Fig. 1. The H_2 reactor and CH_4 reactors A and B comprised the two-stage fermentation systems. The CH_4 reactor C acted as a one-stage fermentation system as a comparison to the two-stage system. The working volumes of H_2 reactor and CH_4 reactors were 3 L and 4 L, respectively. The temperature of the reactors was maintained at 37 ± 1 °C using a temperature controller unit. The volume of the produced biogas from each reactor was measured using a wet tip gas meter which was connected to an automated data acquisition system. The reactor configuration has

been detailed in previous studies [27, 32].

The HRT of the H_2 reactor was set to 4 days. The HRTs of CH_4 reactors A and B were set to 12 days and 24 days, respectively. The HRT of the one-stage CH_4 reactor C was set to 16 days to match the overall HRT of the first two-stage system comprising of the H_2 reactor and the CH_4 reactor A. In a similar fashion, the overall HRT of the second two-stage system comprising of the H_2 reactor and CH_4 reactor B was set to 28 days to match the one in a previous study that investigated the one-stage co-digestion of *L. digitata* and *A. platensis* for methane production [27].

The OLR of the H_2 reactor was increased from 3.0 to 12.0 gVS/L/d with an increment of 3.0 gVS/L/d each time. This was achieved by diluting the algal biomass with a calculated volume of water to keep the HRT unchanged. Every time after feeding, the pH value in H_2 reactor was adjusted to ca. 5.5 using 1 M NaOH solution to ensure the pH did not drop to a level to inhibit hydrogen-producing microbes. The effluent from the H_2 reactor was divided into three parts: the first one as the feedstock for CH_4 reactor A, the second one as the feedstock for CH_4 reactor B, and the third one for analyses. The OLR of CH_4 reactor A ranged from 1.0 to 4.0 gVS/L/d with an increment of 1.0 gVS/L/d each time, whilst that of CH_4 reactor B increased from 0.5 to 2.0 gVS/L/d with an increment of 0.5 gVS/L/d each time. The OLR of the CH_4 reactor C (in the single stage system) started from 1.0 gVS/L/d with an increment of 1.0 gVS/L/d until reactor failure was observed. Each OLR of each reactor was maintained constant for 48 days, which equates to two HRTs of CH_4 reactor C, which had the longest retention time.

2.4 Analytical methods

Total solids (TS) and VS contents of *L. digitata*, *A. platensis*, and inocula were determined using Standard Methods 2540 G [33]. The pH value was measured using a pH meter (Jenway 3510, UK). The ratio of VFAs to total alkalinity (FOS/TAC) was determined based on a two points titration method using 0.1 N H_2SO_4 with end points of pH 5.0 and pH 4.4 [34]. Carbon, hydrogen, and nitrogen contents were determined by an elemental analyser (Exeter Analytical CE 440, UK) and oxygen was calculated as the remaining content of VS. Soluble chemical oxygen demand (sCOD) and total ammoniacal nitrogen (TAN) were measured using Hach Lange cuvette tests (LCK 914 and LCK 303, respectively) and evaluated on a DR3900 Hach Lange Spectrophotometer. Salinity of effluents was determined on a VWR hand held C0310 monitor (VWR international, USA).

The composition of biogas (H_2, CO_2, O_2, N_2, and CH_4) produced in CSTR reactors was determined using a gas chromatograph (GC, Hewlett Packard HP6890, USA) equipped with a Hayesep R packed column and a thermal conductivity detector. The compositions of VFAs in the effluents were determined using a GC (Hewlett Packard HP6890, USA) equipped with a Nukol fused silica capillary column and a flame ionisation detector [32].

2.5 Calculations

The energy values of *L. digitata* and *A. platensis* were calculated using the weight percentages of C, H, N, and O on the basis of the modified Dulong Formula as

shown in Eq. (1) [35]:

$$\text{Energy value of algal biomass (kJ/kg)}=337C+1419(H-0.125O)+23.26N \quad (1)$$

The energy conversion efficiency (ECE) was calculated based on Eq. (2) [36].

$$ECE=\frac{\text{Energy value of } H_2 + \text{Energy value of } CH_4}{\text{Original energy value of algal biomass}}\times 100\% \quad (2)$$

The total chemical oxygen demand (tCOD) of algal biomass was calculated based on the element compositions using Eq. (3) [32]:

$$C_aH_bO_cN_d + (a+\frac{b}{4}-\frac{c}{2}-\frac{3}{4}d)O_2 \rightarrow aCO_2 + \frac{b-3d}{2}H_2O + dNH_3 \quad (3)$$

The acidification yield in the H_2 reactor is defined as the percentage of the COD from VFAs to sCOD as shown in Eq. (4) [32]:

$$\text{Acidification yield}=\frac{COD_{VFAs}}{sCOD_{increase}}\times 100\% \quad (4)$$

The theoretical calculation of biomethane yield was based on the Buswell equation as shown in Eq. (5) [32]:

$$C_aH_bO_cN_d + (a-\frac{b}{4}-\frac{c}{2}+\frac{3}{4}d)H_2O \rightarrow (\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3}{8}d)CH_4 + (\frac{a}{2}-\frac{b}{8}+\frac{c}{4}+\frac{3}{8}d)CO_2 + dNH_3 \quad (5)$$

3. Results and discussion

3.1 Characteristics of algal biomass

Table 1 presents the characteristics of *L. digitata* and *A. platensis* biomass. The macro-algae *L. digitata* was harvested from natural environments in shallow coastal waters, resulting in a lower VS/TS ratio as compare to the artificially cultivated micro-algae *A. platensis* which avoided the significant salt accumulation from

seawater. The harvest timing of September coincided with the peak carbohydrate accumulation in *L. digitata* biomass [5], leading to a high C/N ratio of 26.47. By contrast, the rich proteins in *A. platensis* contributed to the high nitrogen content. This also provided the possibility of mixing the two algal substrates at an appropriate C/N ratio of 20. Moreover, *A. platensis* biomass exhibited higher energy content and theoretical biomethane potential on the basis of elemental composition, despite potential antagonistic effects of recalcitrant organic components on the biodegradability [27]. *L. digitata* biomass is rich in carbohydrates, which generate 20 times higher hydrogen-producing potential than proteins and lipids [40] and as such serve as the major components utilised by the AFB for biohydrogen production. *A. platensis* is rich in proteins and can supply essential nitrogen sources for the anaerobes in both H_2 and CH_4 reactors to maintain effective metabolism [29]. The lipid contents are relatively low in both algal species and are not readily utilised by the AFB for hydrogen production. The lipids, however, can be slowly degraded and further converted to biomethane in the second-stage anaerobic digestion with a longer retention time [22].

3.2 Batch biohydrogen and biomethane potential tests

After the sequential 4-day BHP and 26-day BMP tests using the mixed *L. digitata* and *A. platensis* biomass, a BHP yield of 94.6 mL H_2/gVS and a BMP yield of 309.3 mL CH_4/gVS were recorded (Fig. 2). The BHP yield exceeds the result (60.5 mL H_2/gVS) obtained in a previous study using algal mixture of *L. digitata* and *A.*

293 *platensis* at a C/N ratio of 16.5 [29], indicating the C/N ratio of 20 is preferred during
294 the batch hydrogen fermentation of this specific algal mixture. Moreover, the BHP
295 yield is close to the findings (94.5-97.0 H_2 mL/gVS) of our previous study on batch
296 hydrogen co-fermentation of macro-algae (*L. digitata*) and micro-algae (*Chlorella*
297 *pyrenoidosa* and *Nannochloropsis oceanica*).

298 After hydrogen fermentation, the VFA compositions in the hydrogenogenic
299 effluent were as follows: 0.64 g/L of acetic acid, 0.02 g/L of propionic acid, 0.02 g/L
300 of isobutyric acid, 0.97 g/L of butyric acid, 0.03 g/L of isovaleric acid, and 0.01 g/L
301 of valeric acid. The acetic and butyric acids accounted for 95.1% of the total VFAs,
302 indicating that the predominant metabolic pathways of the AFB during hydrogen
303 fermentation were acetic and butyric routes [22]. As shown in Fig. 2b. during
304 subsequent BMP test, the soluble VFAs that are readily utilised by methanogens
305 contributed to the first peak of biomethane production rate at 6 days, whereas the
306 solid remnants continued to be hydrolysed and resulted in the second peak of
307 biomethane production rate at 12 days. The BMP yield matches that from the one-
308 stage batch anaerobic co-digestion of *L. digitata* and *A. platensis* (311.5 mL
309 CH_4/gVS) achieved by [27]. Although no significant enhancement of BMP yield was
310 obtained, the two-stage batch co-fermentation of *L. digitata* and *A. platensis* secured
311 an overall energy yield of 12.1 kJ/gVS that is 8.5% higher than that from the one-
312 stage biomethane production [27].

313

3.3 Continuous fermentation performances with increasing OLRs

The performance characteristics of all four reactors of the two-stage and one-stage systems over increasing OLRs are summarised in Table 2. The first HRT at each OLR in each reactor was deemed as the acclimatisation period for anaerobic microbes, thus the data in Table 2 are displayed as mean values over the post-first HRT duration of each OLR. Throughout the entire experiment, the TAN concentrations of all CH_4 reactors stayed low, indicating that no ammonia inhibition occurred.

3.3.1 Performance of H_2 reactor

Fig. 3 shows the SHYs of the H_2 reactor with increasing OLRs; Fig 4a shows the compositions of VFAs. At the initial OLR of 3.0 gVS/L/d, the SHYs were quite limited. However, the acidification yield reached 87.5%, indicating a large portion of mixed *L. digitata* and *A. platensis* were utilised by the AFB to maintain basic metabolisms. Thus, the low mean SHY (14.3 mL/gVS) and the high acidification yield at this low OLR indicated that the AFB in H_2 reactor were underfed to some extent. When the OLR increased from 3.0 to 6.0 gVS/L/d, the SHYs drastically increased. Although the SHYs fluctuated between 40.5 and 72.0 mL/gVS over this OLR, an average of 55.3 mL/gVS was achieved, which equates to 58.5% of the BHP yield in the batch trial. As the sCOD of 14.2 g/L at this OLR (6.0 gVS/L/d) was over 2-fold of that (7.0 g/L) at the initial OLR (3.0 gVS/L/d), it could be assumed that the

hydrolysis of mixed algal substrates was even a little bit more efficient. The tVFA also increased to 5254 mg/L, corresponding to an acidification yield of 63.0%. Similarly, the salinity increased by 55.6%, illustrating that this OLR provided excessive biomass supply for the basic metabolisms of AFB and hence more algal substrates were degraded and utilised for hydrogen production.

When the OLR was further lifted from 6.0 to 9.0 gVS/L/d, a sharp drop in hydrogen production was recorded. The mean SHY of 20.4 mL/gVS was 63.1% lower than that at the OLR of 6.0 gVS/L/d. This result was attributed to the accumulation of large quantities of VFAs that inhibited the hydrogen-producing pathways of AFB in the H_2 reactor. The increased loading of algal substrates resulted in sCOD and tVFA values higher by 29.6% and 26.1% in the liquid phase, respectively, whereas the remaining VS in the H_2 reactor (at 9.0 gVS/L/d) increased by 57.5%. As the increase in remaining VS exceeded the increase in sCOD and tVFA, it was assumed that H_2 reactor was overfed and hydrolysis and acidification of loaded algal substrates were limited to some extent. With the OLR further rising to 12.0 gVS/L/d, the average SHY marginally declined to 19.0 mL/gVS. Although the sCOD slightly increased, the tVFA unexpectedly decreased a little bit, leading to a lower acidification yield as compared to that at the OLR of 9.0 gVS/L/d. This also indicated that more algal substrates were fermented through ethanol and lactic acid producing pathways. This was probably ascribed to the enhanced fluctuations of pH values at higher OLRs. With the loading increasing, soluble acidic metabolites accumulated and hence the pH drop became more severe between each feed. The lower pH facilitated the shift of

357 acetic and butyric routes to ethanol and lactic acid producing pathways in the H_2
358 reactor [24, 32, 41].

359 These results suggested that the optimum OLR for continuous biohydrogen
360 production through co-fermentation of macro-algae *L. digitata* and micro-algae *A.*
361 *platensis* was 6.0 gVS/L/d in the H_2 reactor. The insufficient biomass supply at lower
362 OLR failed to provide essential feedstock for the AFB to produce hydrogen, whereas
363 the overfeeding of algae at higher OLRs resulted in the accumulation of VFAs which
364 in turn suppressed the hydrogen-producing metabolisms.
365

366 **3.3.2 Performance of CH_4 reactors A and B**

367 The SMYs of CH_4 reactors A and B of the two-stage system and the variation
368 trends of tVFA and FOS/TAC values over increasing OLRs are illustrated in Fig. 3
369 and Fig. 5, respectively. At the initial OLR of 1.0 gVS/L/d, CH_4 reactor A performed
370 best with an average SMY of 265.5 mL/gVS which accounted for 85.8% of the BMP
371 value in the batch trial. The sCOD and tVFA were low at 0.6 g/L and 354 mg/L,
372 respectively, indicating that most of the soluble metabolites produced via first-stage
373 dark hydrogen fermentation were utilised by the microbes in CH_4 reactor A. The
374 FOS/TAC value was low (0.22) as well. When the OLR increased to 2.0 gVS/L/d, the
375 average SMY slightly decreased to 245.0 mL/gVS, signifying 79.2% of the BMP
376 yield. The low FOS/TAC value of 0.17 ensured the process stability of second-stage
377 anaerobic digestion. Under the conditions of higher sCOD and tVFA inputs from

effluents of the H_2 reactor, the sCOD and tVFA values of CH_4 reactor A remained almost as low as those at the previous OLR of 1.0 gVS/L/d, resulting in even higher sCOD and tVFA destruction efficiencies (93.7% and 93.3%, respectively). The continuous increase of OLR from 2.0 to 3.0 gVS/L/d further led to a 9.4% drop in SMY. Although the FOS/TAC value remained within a suitable range, both the VFAs and sCOD increased. The average tVFA value of 877 mg/L was not high, however, the variation trend shown in Fig. 5 implied that the accumulation of VFAs was in progress. Especially as shown in Fig. 4b, the content of propionic acid in CH_4 reactor A significantly increased at 3.0 gVS/L/d as compared to the lower loading rates. The accumulation of propionic acid in the digester is always deemed as an indicator of impending anaerobic digestion failure [42, 43]. At the maximum OLR of 4.0 gVS/L/d, a notable reduction in SMY was recorded: the SMY of 174.0 mL/gVS was lower than that at 3.0 gVS/L/d by 24.1% and only equivalent to 65.5% of the highest one obtained at 1.0 gVS/L/d. The sCOD and tVFA further accumulated in CH_4 reactor A. The average FOS/TAC value increased to 0.27 and the variation trend shown in Fig. 4 suggested that the FOS/TAC of CH_4 reactor A was rising towards the threshold value. Fig. 4b shows that the propionic acid concentration further increased to 775 mg/L and almost all the iso-acids were higher, illustrating that the process instability of CH_4 reactor A caused by the overloading of mixed algal biomass was in progress [42]. The struggling of CH_4 reactor A at higher OLRs could be associated with the inability of the microbial community to acclimatise to such a high loading in a short HRT of 12 days. This may have resulted in washout of microbial community.

Since CH_4 reactors A and B shared the same feedstock origin (effluent from H_2 reactor), the 2-fold HRT of CH_4 reactor B led to lower OLRs which equates to half of those of CH_4 reactor A. The FOS/TAC values remained low (0.17-0.19) throughout the entire continuous experiments, indicating that a more stable second-stage anaerobic digestion process was ensured by the longer HRT and lower OLRs of CH_4 reactor B as compared to CH_4 reactor A. Although the SMYs were marginally lower than the highest one obtained in CH_4 reactor A, the average values in CH_4 reactor B were less affected by the increasing OLR from 0.5 to 2.0 gVS/L/d and remained within a reasonable range of 223.8-242.5 mL/gVS signifying 72.4-78.4% of the BMP value and 46.7-50.6% of the theoretical methane yield. The sCOD and tVFA stayed low over increasing OLRs, leading to the high sCOD (88.6-95.1%) and tVFA (92.2-95.6%) destruction efficiencies. However, the highest average sCOD (2.2 g/L) and tVFA (551 mg/L) recorded at the maximum OLR of 2.0 gVS/L/d were both higher than those in CH_4 reactor A at the same OLR. This was caused by the feedstock sourced from the effluent of the H_2 reactor at various OLRs. At an OLR of 2.0 gVS/L/d, the feedstock loaded into CH_4 reactor B was obtained from the effluent of the H_2 reactor at an OLR of 12.0 gVS/L/d, whilst the one loaded into CH_4 reactor B was originated from the effluent of the H_2 reactor at an OLR of 6.0 gVS/L/d. The sCOD and tVFA values of the former was markedly higher than the latter, resulting in a comparatively more severe impact on the second-stage anaerobic digestion process. Nonetheless, Fig. 4c reveals that no accumulation of propionic acid or iso-acids in CH_4 reactor B were observed at an OLR of 2.0 gVS/L/d, demonstrating that no

422 inhibition of methanogens or anaerobic digestion process failure was evident.

423 Overall, considering SMY, treating capacity, and process stability, an OLR of
424 2.0 gVS/L/d was shown to be optimal for CH_4 reactor A at a fixed HRT of 12 days.

425

426 **3.3.3 Performance of CH_4 reactor C**

427 The SMYs of CH_4 reactor C of the one-stage system are shown in Fig. 3. With
428 the OLR increasing from 1.0 to 3.0 gVS/L/d, the average SMYs gradually decreased
429 from 204.5 to 72.2 mL/gVS. As shown in Fig. 5, the VFAs accumulated and the
430 FOS/TAC values rose along with the increasing OLR, indicating that the buffer
431 capacity in the CH_4 reactor C was strongly negatively correlated with OLR in this
432 one-stage system. At the initial OLR of 1.0 gVS/L/d, the tVFA already reached 1287
433 mg/L and the VFA composition in Fig. 4d revealed that propionic acid accounted for
434 65.6% of the tVFA. This phenomenon of propionic acid accumulation was similar to
435 that obtained in the CH_4 reactor A at the maximum OLR of 4.0 gVS/L/d, signifying
436 that the process instability of one-stage anaerobic co-digestion was triggered. When
437 the OLR rose to 2.0 gVS/L/d, a remarkable surge in VFAs was noted: the tVFA
438 concentration of 6593 mg/L was even close to that in the H_2 reactor at 9.0 gVS/L/d. It
439 was assumed that the methanogens in CH_4 reactor C suffered severe inhibition under
440 such acidic condition. When the OLR further increased to 3.0 gVS/L/d, the sCOD
441 increased by 110.7%, whereas the tVFA slightly decreased instead, indicating that the
442 acidification process was impaired even though the hydrolysis was efficient. In

addition, the enhancements of propionic, butyric, and longer-chain acids and little accumulation of acetic acid were recorded in Fig. 4d. These results suggested that the microbial community was highly affected: the activity of acetogens and methanogens were inhibited to a great extent. Furthermore, the salinity in CH_4 reactor C amounted to 13.3 g/kg, which was far higher than the highest ones obtained in CH_4 reactors B and C during the entire experiment. Although small concentrations of sodium ions (100-350 mg/L) are supposed to be essential for the maintenance of healthy metabolism of the microbes in anaerobic digesters [44], the enhanced osmotic pressure caused by the remarkably high salinity can inhibit microbial activity and even lead to dehydration of microbes [23]. Luo et al. [45] investigated the effects of saline adaptation on anaerobic digestion of sludge and observed that salinity levels higher than 8.7 g/kg impaired the methane production. On the other hand, Tabassum et al. [46] demonstrated acclimatisation to salinity levels of the order of 14 g/L in mono-digestion of farm cultivated *S. latissima* at an OLR of 4.0 kgVS/m^3/d. The high salinity levels recorded here of 13.3g/kg at an OLR of 3.0 gVS/L/d will have some inhibitory effects on the microbial consortium in CH_4 reactor C. Although the gas production did not thoroughly stop, the failure of CH_4 reactor C was inevitable.

In a previous study, [27] conducted continuous one-stage anaerobic co-digestion of *L. digitata* and *A. platensis* based on a C/N ratio of 25 at a long HRT of 28 days. A high OLR of 4.0 gVS/L/d was shown to be tolerable for the CH_4 reactor and an SMY of 259.6 mL/gVS was recorded. Despite the different seed inocula and minor variation in C/N ratios, the significant reduction in HRT (28 days as compared to 16

days here) was assumed to be the key influencing factor between these two one-stage systems. It is suggested that an HRT of 16 days did not supply sufficient time for acclimatisation and enrichment of the microbial consortium in the CH_4 reactor C and led to washout of microbes, accumulation of VFAs, and inhibition of methanogenesis.

3.4 Comparisons between two-stage and one-stage fermentation performances

The two-stage system comprising of the H_2 reactor and the CH_4 reactor A and the one-stage system of CH_4 reactor C shared comparable operational parameters such as overall HRT (16 days), OLR, temperature (37 ± 1 °C), and initial seed inoculum for methane production. At an OLR of 6.0 gVS/L/d, the highest average SHY of 55.3 mL/gVS, which equates to 58.5% of the BHP yield in batch trail, was obtained in the first-stage dark hydrogen fermentation. In the second-stage anaerobic digestion, the average SMY of 245.0 mL/gVS equivalent to 79.2% of the BMP value was achieved in CH_4 reactor A at a corresponding OLR of 2.0 gVS/L/d, and process stability was secured. The two-stage system effected an energy yield of 9.4 kJ/gVS and the ECE amounted to 51.0%. The energy yield of the continuous two-stage system was 22.3% lower than the batch trial. This is expected due to the disadvantages of shorter retention time (16 days in two-stage versus 30 days for batch) and the larger reactor with less efficient mixing conditions. By contrast, in the one-stage system, the CH_4 reactor C recorded its highest SMY of only 204.5 mL/gVS at the initial OLR of 1.0 gVS/L/d. The energy yield and ECE were lower at 7.3 kJ/gVS and 39.8%, respectively. Even at this low OLR, a certain degree of VFA accumulation was

observed. When the OLR rose to 3.0 gVS/L/d, the process instability of one-stage anaerobic co-digestion of *L. digitata* and *A. platensis* became more obvious. Therefore, the two-stage system prevailed in both energy production from mixed algal feedstock and treating capacity as compared to one-stage system at a fixed HRT of 16 days. Even if the energy content in produced hydrogen was nearly negligible, the first-stage dark hydrogen fermentation would serve as an optimised hydrolysis and acidification method pretreating the mixed algal feedstock. Similar results were reported by [26, 32] utilising grass and food waste in continuous two-stage systems. To sum up, the technical feasibility of two-stage co-fermentation of *L. digitata* and *A. platensis* biomass has been proven, and several operational parameters have been assessed via this 32-week long experimentation, thus mitigating the gaps between the fundamental innovations obtained by the small-scale batch co-fermentation and the potential commercial deployment of algal biofuel systems in future.

Although positive results on two-stage continuous hydrogen and methane co-production using mixed *L. digitata* and *A. platensis* have been achieved in this study, some issues are still noteworthy. The C/N ratio was adjusted to 20 in the mixture of macro- and micro-algae, however, the TAN levels stayed low in all four reactors throughout the entire continuous experiment, indicating that the hydrolysis or degradation of nitrogen-rich micro-algae biomass may have been somewhat limited, especially in a short HRT of 16 days. This was probably ascribed to limited degradation of untreated *A. platensis* due to its recalcitrant cell wall structures. The slow or limited utilisation of micro-algae biomass further restricted the

509 fermentation/digestion process and also explained why the longer HRT in CH_4 reactor
510 B and in the previous study [27] could ensure a more stable process. Therefore, to
511 overcome this drawback, pretreatment of micro-algae and even macro-algae to
512 facilitate the solubilisation and hydrolysis of feedstock is a promising option for a
513 stable continuous fermentation/digestion process in future study.
514

515 **3.5 Comparison between results of this study and relevant literature**

516 To the best of our knowledge, most of the studies on biohydrogen and
517 biomethane production from either macro- or micro-algae biomass were conducted in
518 batch trials [23, 30]. The data on long term continuous fermentation of algae are
519 relatively limited. A comparison between the results of continuous fermentative
520 gaseous biofuel production from algal biomass and other co-substrates in this study
521 and the state of the art in the literatures is summarised in Table 3. Tabassum et al. [46]
522 found that a mixed feedstock of 66.6% macro-algae (*L. digitata* or *S. latissima*) and
523 33.3% dairy slurry was optimal to obtain a maximum biomethane production
524 efficiency during continuous anaerobic co-digestion. The energy yields (9.0-9.3
525 kJ/gVS) were close to that obtained in this study. Allen et al. [47] suggested for the
526 green macro-algae (*Ulva lactuca*) that the optimal mixture in long term continuous
527 digestion would be 25% macro-algae and 75% dairy slurry; this resulted in an SMY
528 of 170 mL/gVS, equivalent to 95% of the BMP value. These differences are attributed
529 to the significant variation in biological characteristics of different macro-algal
530 species. The green seaweed *U. lactuca* typically has a C/N ratio below 10 and as such

531 needs to be co-digested with a carbohydrate-rich co-substrate to increase the C/N ratio
532 for better digestibility. The carbohydrate-rich brown seaweeds *L. digitata* and *S.*
533 *latissima* have high C/N ratios (>25) when they are ripest in late summer [46].
534 Similarly, the protein-rich Taihu blue algae with a low C/N ratio of 6.1 resulted in an
535 SMY of 160 mL/gVS, whereas the mixture of Taihu blue algae and carbohydrate-rich
536 corn straw with a C/N ratio of 20 resulted in an increase in SMY of 46% [48].
537 Herrmann et al. [27] also used micro-algae *A. platensis* as a nitrogen-rich additive to
538 macro-algae *L. digitata* for adjusting the C/N to 25. Compared with the results
539 obtained in the one-stage reactor in this study, the longer HRT (28 days) allowed a
540 higher OLR (4.0 gVS/L/d) with a stable process and a higher SMY. All the above
541 studies were conducted in a one-stage system; only one previous study investigated
542 two-stage continuous fermentation of macro-algae *L. digitata* [49]. The two-stage
543 fermentation system outperformed one-stage system with a higher energy yield in a
544 shorter overall HRT [49]. This finding was consistent with the output of this study.
545 The optimal HRT, OLR, and biofuel yield varied between the studies due to different
546 experimental configurations, different sources of inocula, and different algal
547 feedstocks. However, the results showed similarities in C/N ratios, and the
548 improvements in energy return and process stability.
549

550 **3.6 Gross energy potential from algal mixture**

551 In this study, the major component in the algal mixture is macro-algae *L.*
552 *digitata*, which accounts for 94% of the VS. The co-substrate micro-algae may be

553 considered as a nitrogen-rich additive. Therefore, the gross energy potential from this
554 mixed algal feedstock is heavily associated with the *L. digitata* biomass resource.
555 Nonetheless, the definite data on the annual yields of seaweed per hectare are not
556 available because of a series of variations, such as algal species, locations, harvesting
557 times, etc [44]. According to a latest report of International Energy Agency
558 Bioenergy, the yields of *L. digitata* cultivated using advanced textiles in open sea
559 reached 16 kg/m^2, equivalent to 160 tons wet weight per hectare per year (t
560 wwt/ha/yr) [50]. Under this scenario, based on the energy yield of 9.4 kJ/gVS in the
561 two-stage continuous co-fermentation system, the gross energy potential is calculated
562 to be 213 GJ/ha/yr. This value is comparable with the gross energy yields of
563 biomethane from terrestrial crops, such as maize (217 GJ/ha/yr), fodder beet (250
564 GJ/ha/yr), and grass (163 GJ/ha/yr) [51]. The advantages of algae cultivation, are that
565 as an advanced third generation biofuel there is no requirement for arable land, the
566 fuel is outside the food-or-fuel debate, and it is an attractive process for countries with
567 long coastlines [44]. For example, in China, Shandong Province is one of the biggest
568 mariculture bases, and macro-algae is one of the major products [52]. Meanwhile, a
569 modern microalgal cultivation plant equipped with large raceway ponds has been
570 constructed in Penglai City, Shandong Province. Seawater is used as basic culture
571 solution, and flue gas from a coal-fired power plant is used as the CO_2 source [53,
572 54]. These examples in the literature indicate that both macro-algae and micro-algae
573 can be grown in the same place, making the combined use of the macro- and micro-
574 algae reasonable and feasible. In addition, integrated multi-trophic aquaculture

(coupling seaweed production with fish farms) captures nutrients from fish excrement enhancing seaweed growth and water quality [5], and leading to promotion of industrial scale advanced gaseous biofuel production from algal biomass.

4. Conclusions

A continuous two-stage system involving dark hydrogen fermentation and anaerobic fermentation of mixed macro-algae and micro-algae at a C/N ratio of 20 was shown to be feasible with an overall ECE of 51.0%. The short HRT (16 days) allowed an efficient fermentation process in the H_2 reactor at 6.0 gVS/L/d and a stable digestion process in the CH_4 reactor at a corresponding OLR of 2.0 gVS/L/d. In contrast to the one-stage system, the first-stage dark hydrogen fermentation in the two-stage system optimised hydrolysis and acidification of algal mixtures, hence facilitating improved methane production and process stability in second-stage anaerobic digestion. The gross energy potential of 213 GJ/ha/yr makes this algal mixture comparable with terrestrial crops in gaseous biofuel production while removing any land use implications.

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601

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List of figures and tables:

779 **Table 1 Characteristics of algal biomass**

Parameter	*Laminaria digitata*	*Arthrospira platensis*	Mixed *Laminaria digitata* and *Arthrospira platensis*
Proximate analysis			
Moisture (wt%)	81.87	6.40	81.16
TS (wt%)	18.13	93.60	18.84
VS (wt%)	13.31	86.77	14.01
VS/TS (%)	73.44	92.70	74.34
Ultimate analysis			
C (TS%)	36.08	49.27	36.70
H (TS%)	4.67	6.58	4.76
O (TS%)	31.32	25.48	1.84
N (TS%)	1.36	11.38	31.05
C/N ratio	26.47	4.33	20.00
Biological analysis			
Proteins (TS%)	7.32[a]	71.13[a]	10.32
Lipids (TS%)	0.92[b]	5.00[c]	1.11
Carbohydrates (TS%)	65.20[d]	16.57[d]	62.91
Energy value (kJ/gVS)	18.1	23.4	18.4
tCOD (gCOD/gVS)	1.36	1.50	1.37
Theoretical biomethane yield (mL/gVS)	476.3	525.2	479.2

780 a: The contents of proteins are calculated by multiplying the nitrogen contents by a
781 factor of 5.38 for brown seaweeds [37] and 6.25 for microalgae [38].
782 b: The lipid content of *Laminaria sp.* is suggested to be 0.92% of the dry weight by
783 Sánchez-Machado et al. [39].
784 c: The lipid content of *Arthrospira sp.* is suggested to be 5% of the dry weight by
785 Dismukes et al. [6].
786 d: It is assumed that the sum of proteins, lipids, carbohydrates equates to the VS of
787 algal biomass.

788 **Table 2 Summary of results from two-stage and one-stage co-fermentation of *L. digitata* and *A. platensis* (mean values of post-first HRT**
789 **for each OLR)**

	H_2 reactor				CH_4 reactor A				CH_4 reactor B				CH_4 reactor C		
HRT (days)	4				12				24				16		
OLR (gVS/L/d)	3.0	6.0	9.0	12.0	1.0	2.0	3.0	4.0	0.5	1.0	1.5	2.0	1.0	2.0	3.0
SHY (mL/gVS)	14.3	55.3	20.4	19.0	/	/	/	/	/	/	/	/	/	/	/
SMY (mL/gVS)	/	/	/	/	265.5	245.0	229.1	174.0	242.5	228.9	223.8	236.5	204.5	134.8	72.2
FOS/TAC	/	/	/	/	0.22	0.17	0.21	0.27	0.19	0.17	0.17	0.17	0.61	1.03	1.68
TAN (mg/L)	7	2	4	5	216	148	251	269	281	197	290	279	95	43	158
TS (g/kg)	14.3	23.8	37.9	45.3	11.8	12.8	18.9	23.9	17.3	13.3	19.4	23.5	12.2	26.3	47.6
VS (g/kg)	9.4	15.3	24.1	29.5	5.6	5.4	6.7	9.3	8.7	5.4	7.3	8.0	6.7	12.0	22.5
sCOD (g/L)	7.0	14.2	18.4	21.5	0.6	0.9	2.3	5.2	0.8	0.7	1.4	2.2	2.5	10.3	21.7
tVFA (mg/L)	3776	5254	6626	6587	354	349	877	1365	243	287	279	551	1287	6593	5982
COD_{VFAs} (g/L)	6.2	8.9	11.4	11.5	0.6	0.6	1.4	2.1	0.4	0.5	0.5	0.9	2.1	8.9	10.5
Acidification yield (%)	87.5	63.0	62.2	53.5	/	/	/	/	/	/	/	/	/	/	/
Salinity (g/kg)	3.6	5.6	6.5	5.8	4.6	6.4	8.1	5.6	6.6	6.5	8.1	7.7	4.5	9.7	13.3
Energy yield (kJ/gVS)	0.2	0.6	0.2	0.2	9.5	8.8	8.2	6.2	8.7	8.2	8.0	8.5	7.3	4.8	2.6
ECE (%)	0.8	3.3	1.2	1.1	51.7	47.7	44.6	33.9	47.2	44.6	43.6	46.0	39.8	26.2	14.1

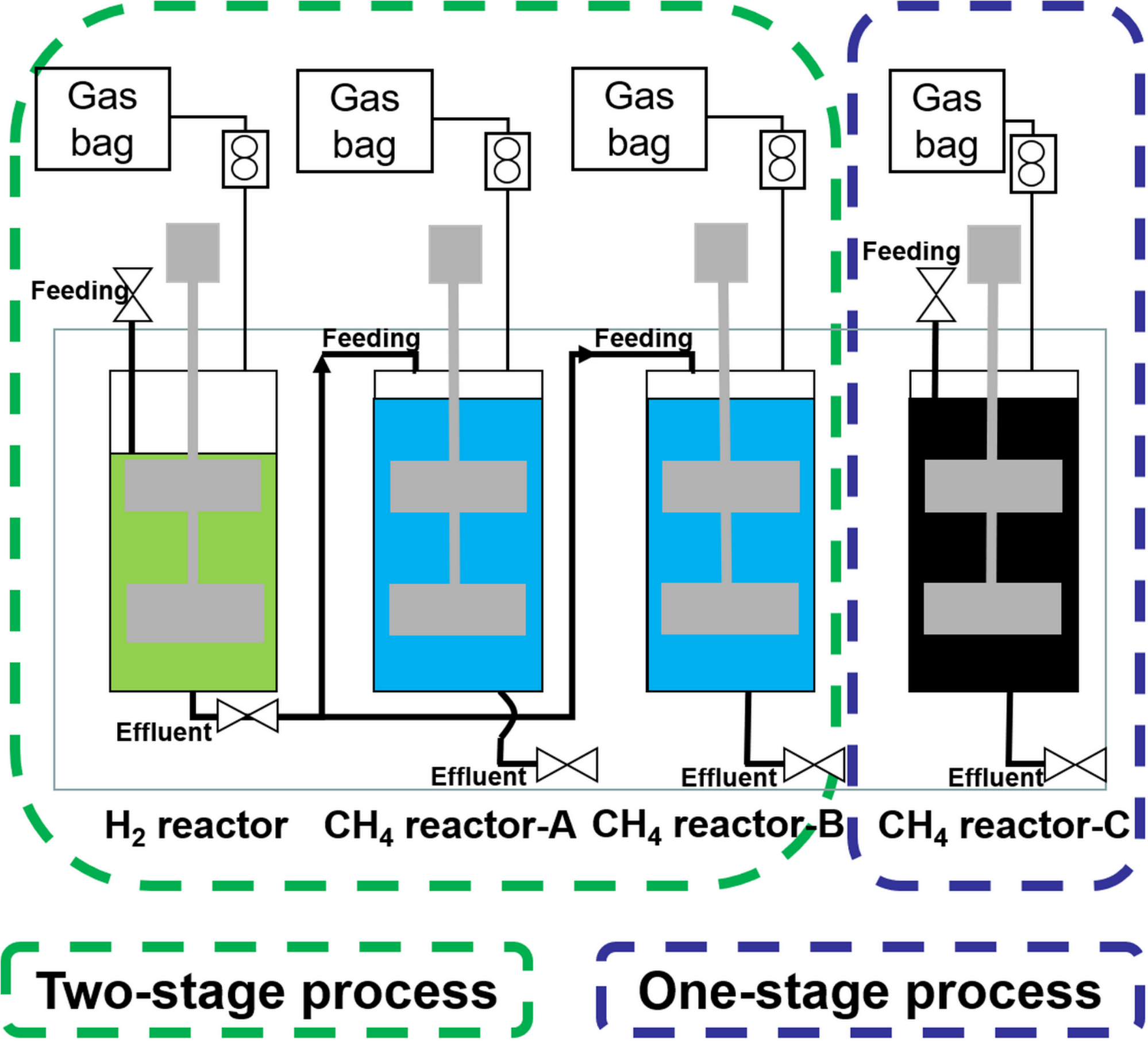
790
791

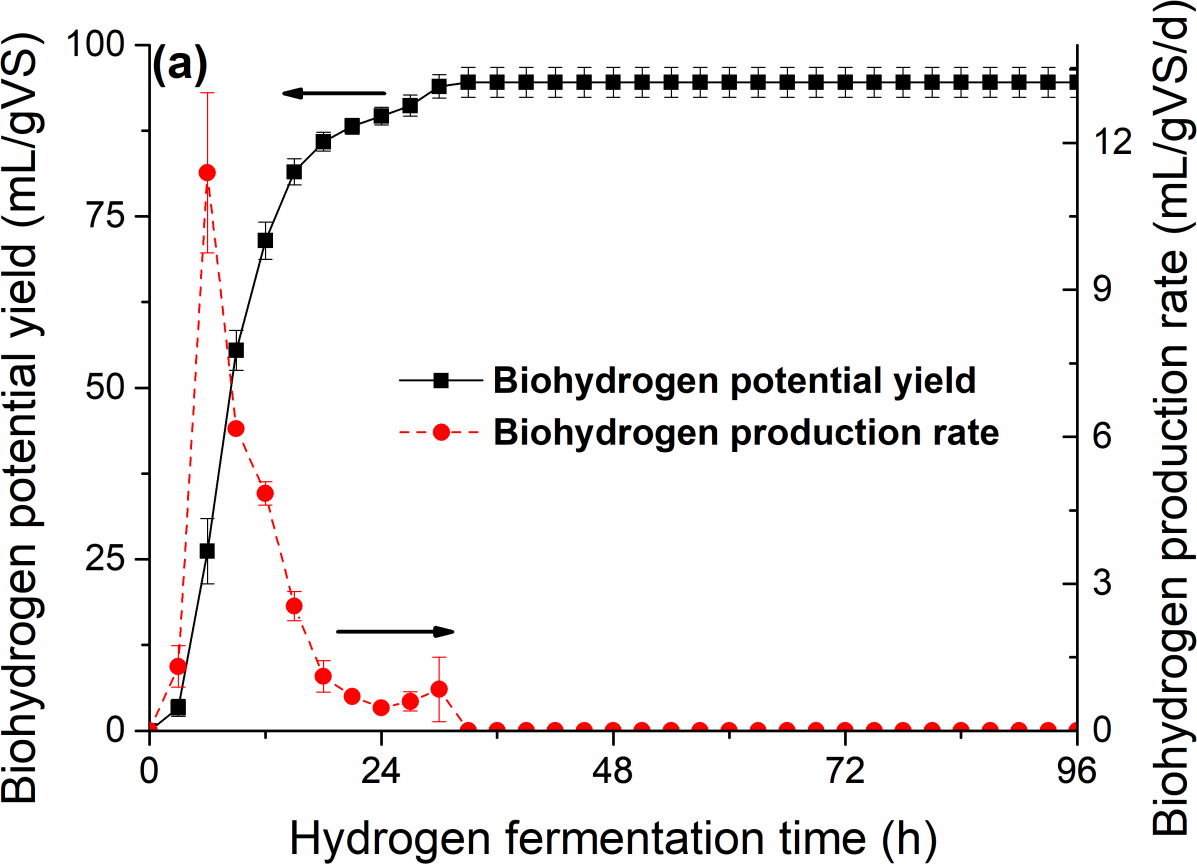
792 **Table 3 Comparison between the results in this study and relevant literatures on continuous fermentative gaseous biofuel production**
793 **from algal biomass**

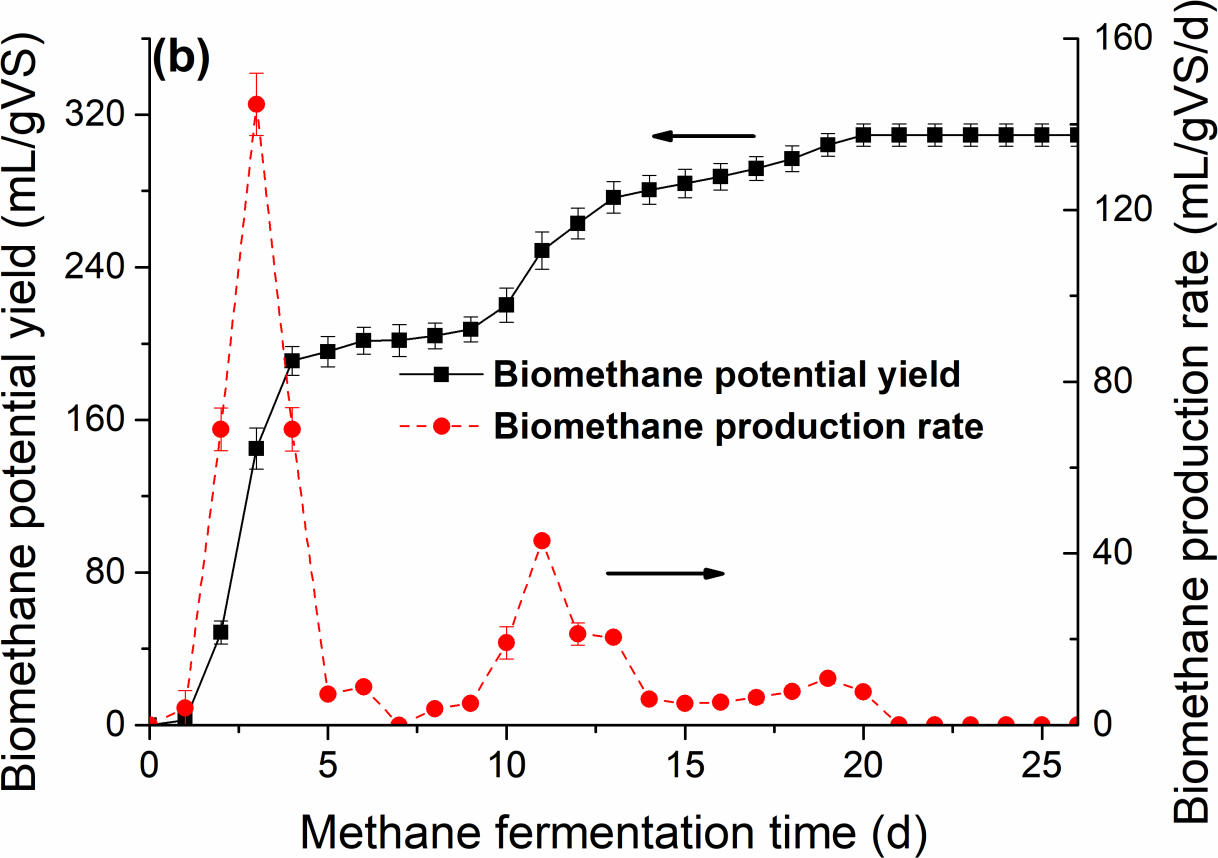
Algal species	Co-substrate	Fermentation type	HRT (d)	OLR (gVS/L/d)	SHY (mL/gVS)	SMY (mL/gVS)	C/N ratio	Energy yield (kJ/gVS)	Reference
Laminaria digitata	Dairy slurry	One-stage CH_4 fermentation	18	4.0	/	261	23.4	9.3	[46]
Saccharina latissima			13	4.0	/	252	15.7	9.0	
Ulva lactuca	Dairy slurry	One-stage CH_4 fermentation	42	2.5	/	170	16.6	6.1	[47]
Taihu blue algae	/	One-stage CH_4 fermentation	10	6.0	/	160	6.1	5.7	[48]
	Corn straw			6.0	/	234	20	8.4	
Laminaria digitata	*Arthrospira platensis*	One-stage CH_4 fermentation	28	4.0	/	259.6	25	9.3	[27]
Laminaria digitata	/	One-stage CH_4 fermentation	24	2.4	/	221	27.3	7.9	[49]
		Two-stage H_2 + CH_4 fermentation	4 (H_2) + 14 (CH_4)	12 (H_2) + 3.43 (CH_4)	26	234		8.7	
Laminaria digitata	*Arthrospira platensis*	One-stage CH_4 fermentation	16	1.0	/	204.5	20	7.3	This study
		Two-stage H_2 + CH_4 fermentation	4 (H_2) + 12 (CH_4)	6.0 (H_2) + 2.0 (CH_4)	55.3	245.0		9.4	
		Two-stage H_2 + CH_4 fermentation	4 (H_2) + 24 (CH_4)	12.0 (H_2) + 2.0 (CH_4)	19.0	236.5		8.7	

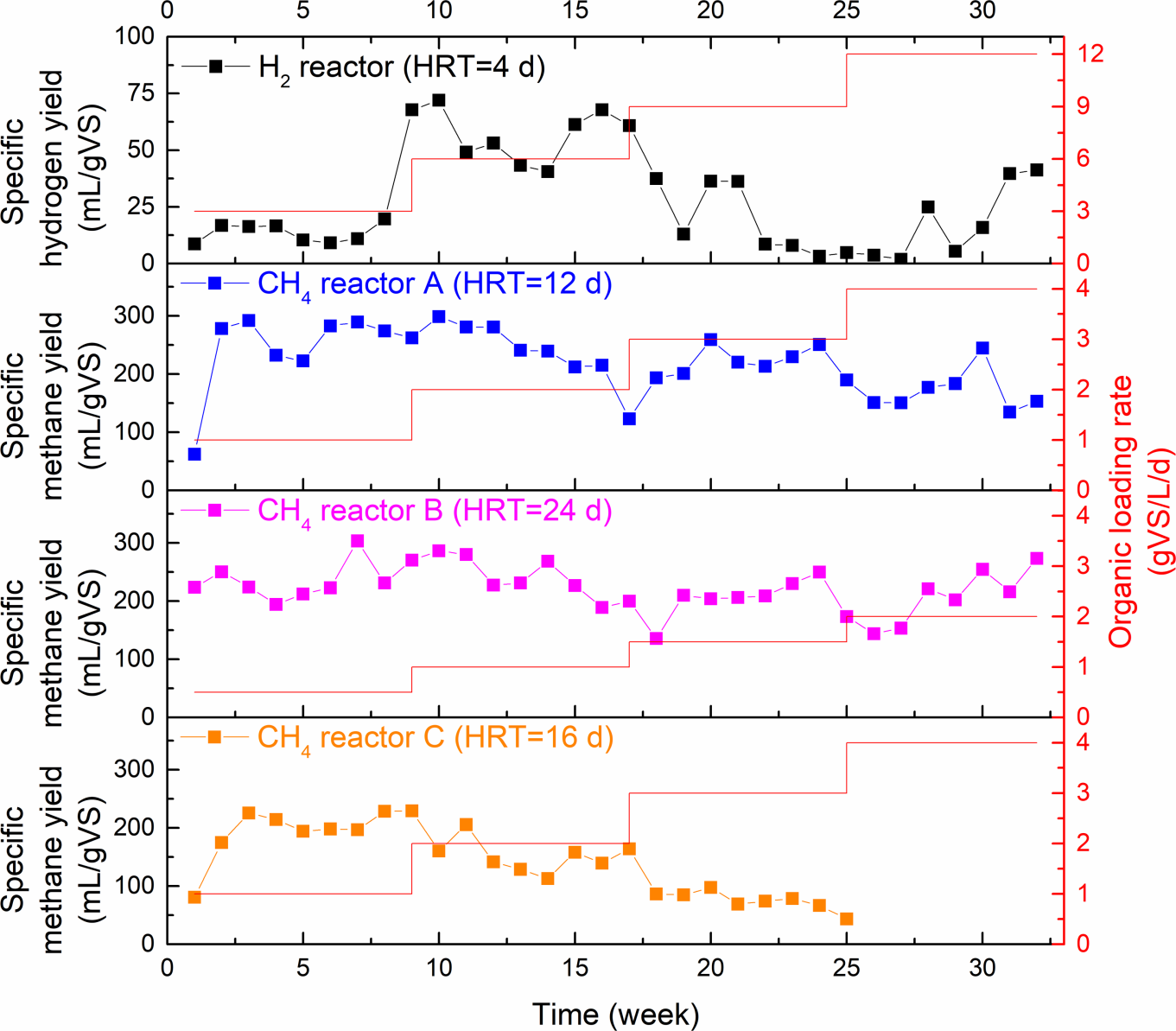
794

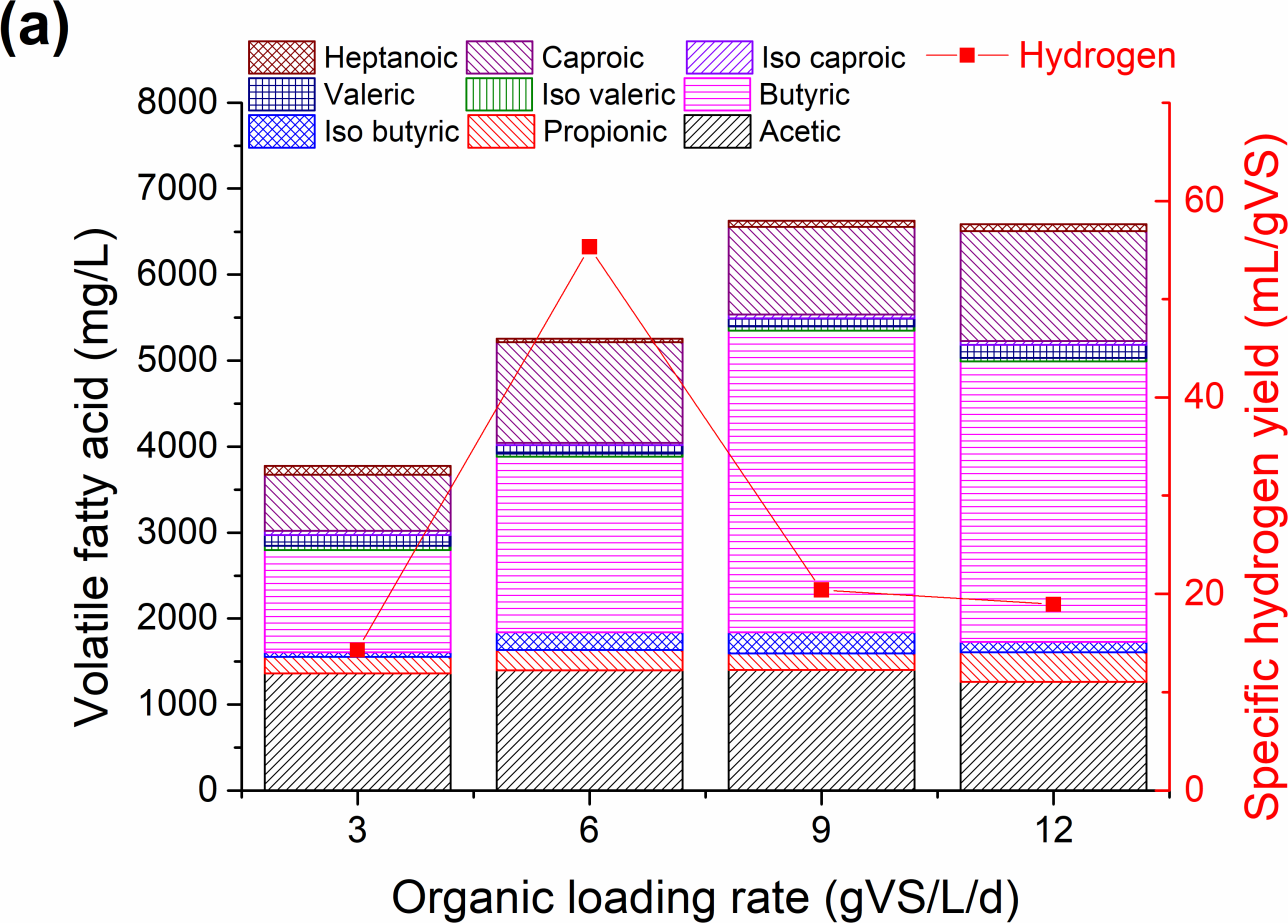
- Two-stage continuous co-fermentation of macro- and micro-algae was investigated.
- Optimum H_2 production was observed at an organic loading rate (OLR) of 6.0 gVS/L/d.
- Second-stage CH_4 production was stable at a corresponding OLR of 2.0 gVS/L/d.
- The two-stage system gave an energy yield of 9.4 kJ/gVS at a retention time of 16 d.
- Gross energy potential of this algal mixture may reach 213 GJ/ha/yr.

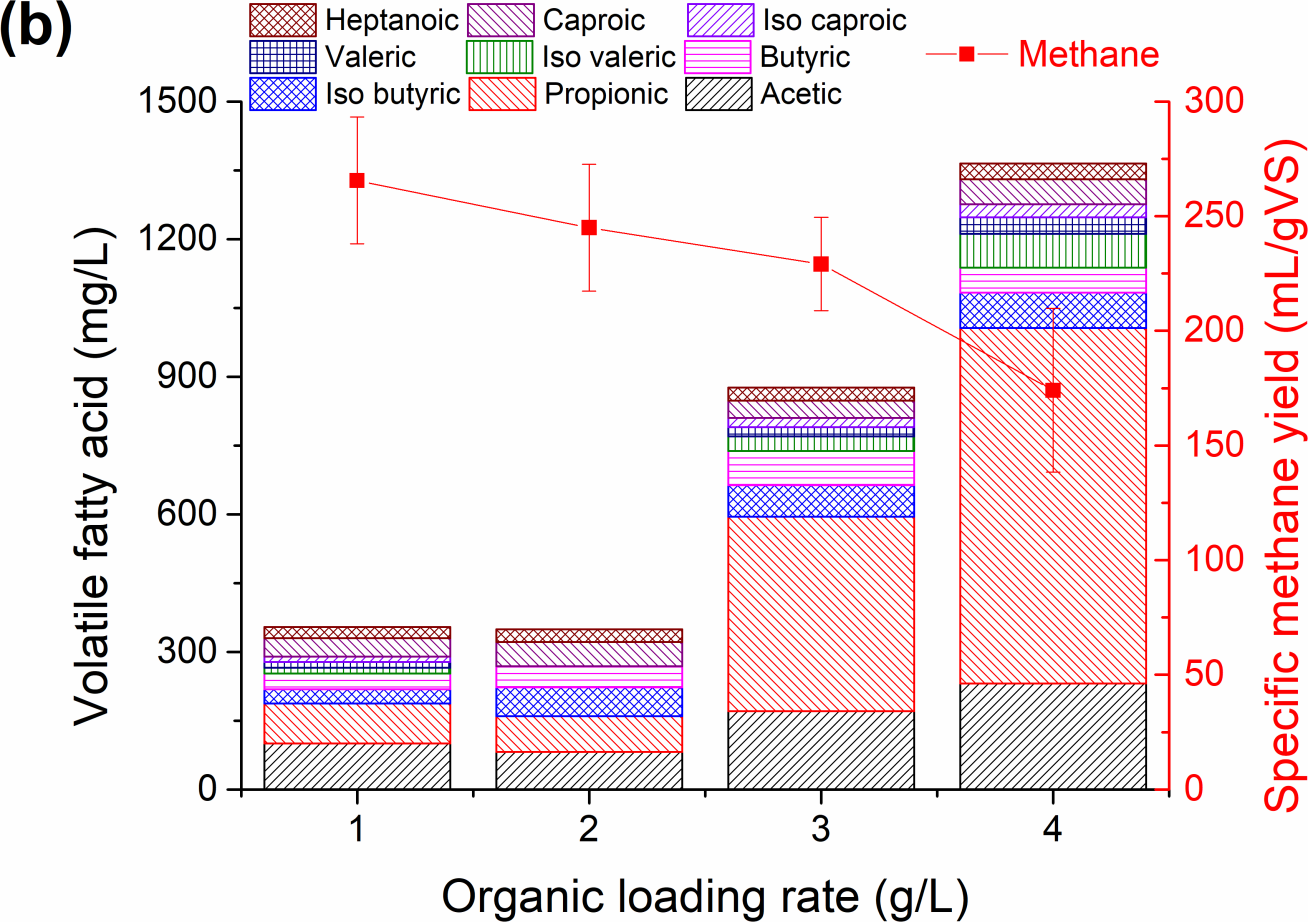

Gas bag
Gas bag
Gas bag
Gas bag
Feeding
Feeding
Feeding
Feeding
Effluent
Effluent
Effluent
Effluent
H2 reactor
CH4 reactor-A
CH4 reactor-B
CH4 reactor-C
Two-stage process
One-stage process

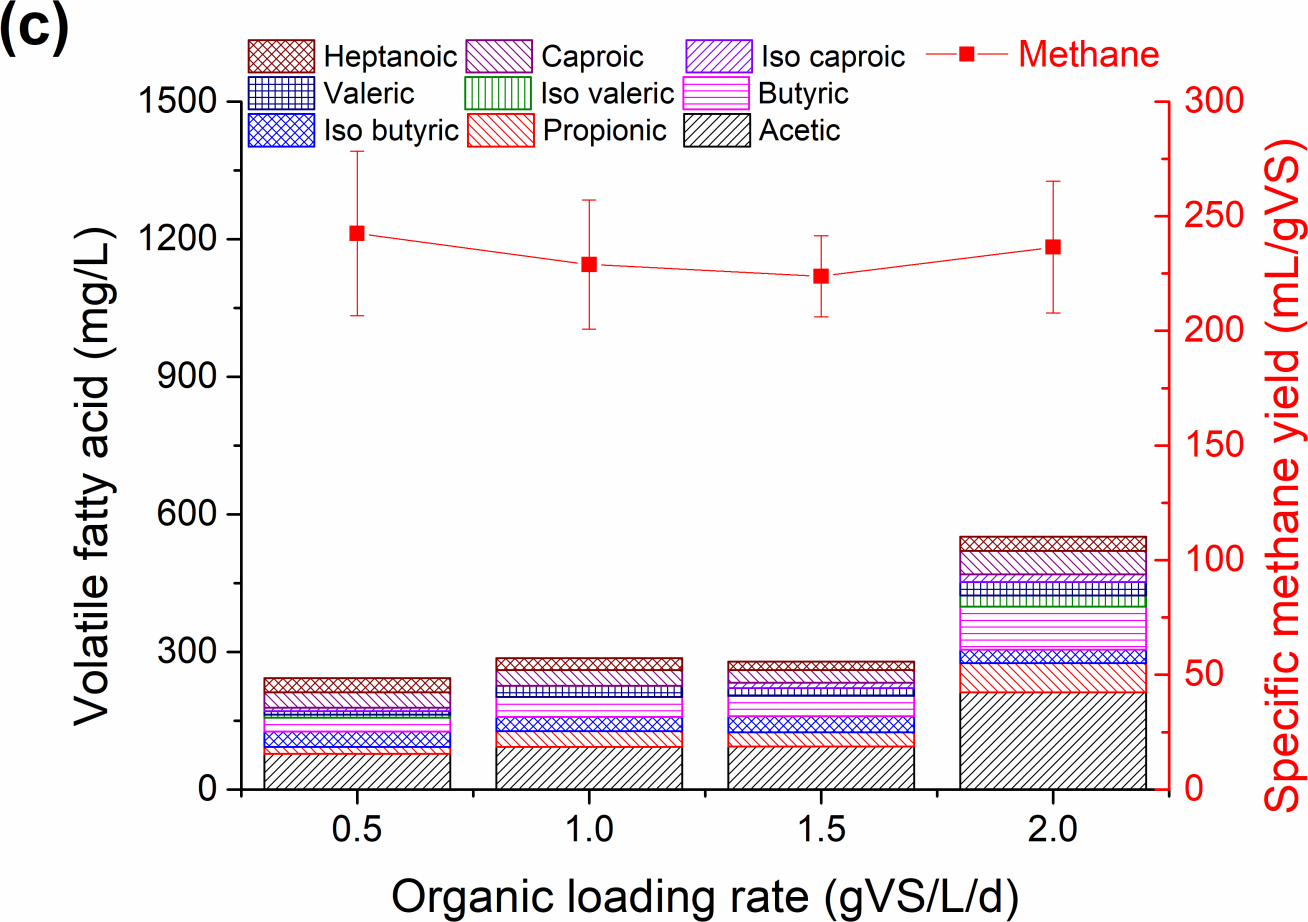

(a)
Biohydrogen potential yield
Biohydrogen production rate
Biohydrogen potential yield (mL/gVS)
Biohydrogen production rate (mL/gVS/d)
Hydrogen fermentation time (h)
0
25
50
75
100
0
3
6
9
12
0
24
48
72
96

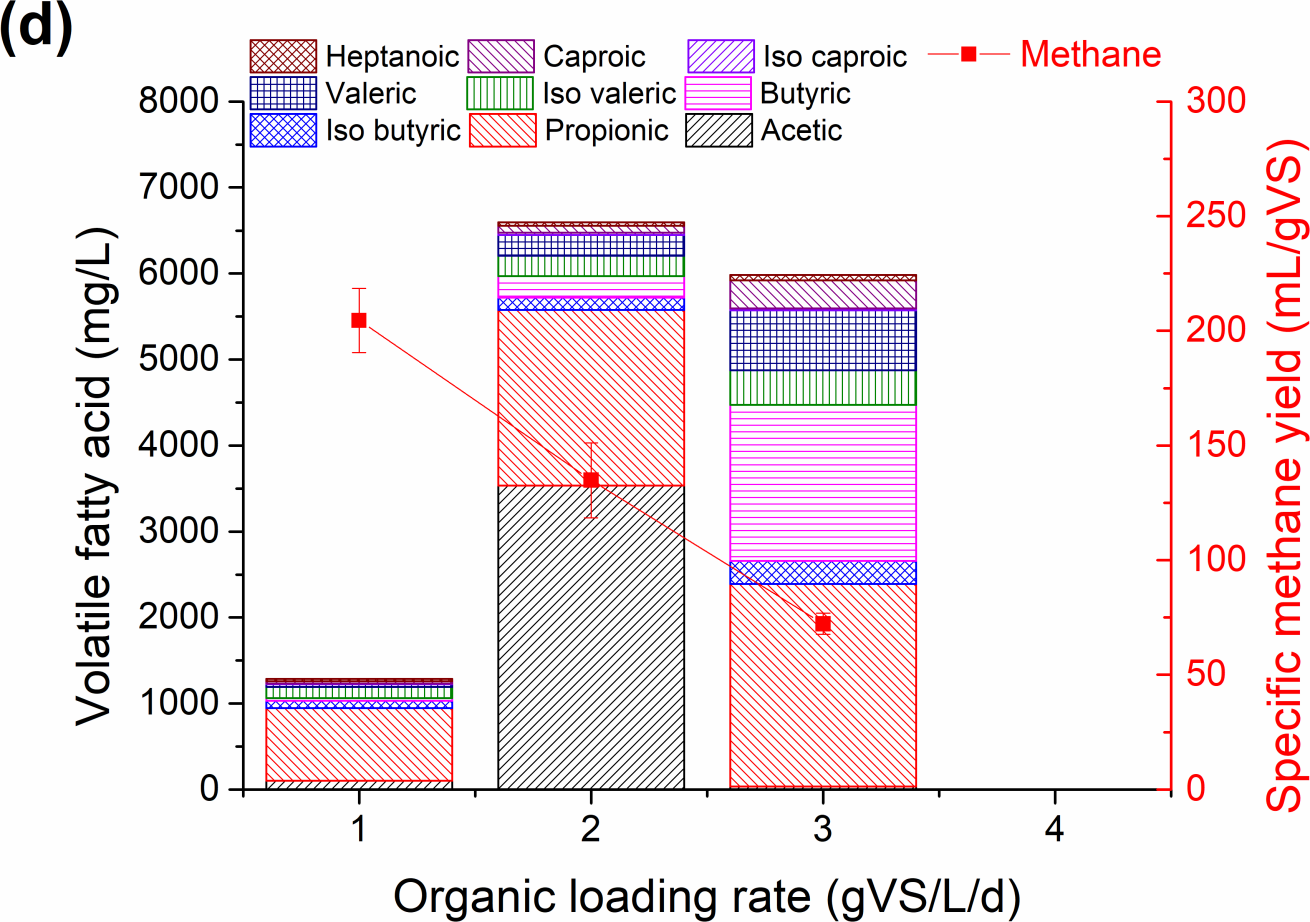

(b)
Biomethane potential yield (mL/gVS)
Biomethane production rate (mL/gVS/d)
Methane fermentation time (d)
Biomethane potential yield
Biomethane production rate
0
80
160
240
320
0
40
80
120
160
0
5
10
15
20
25

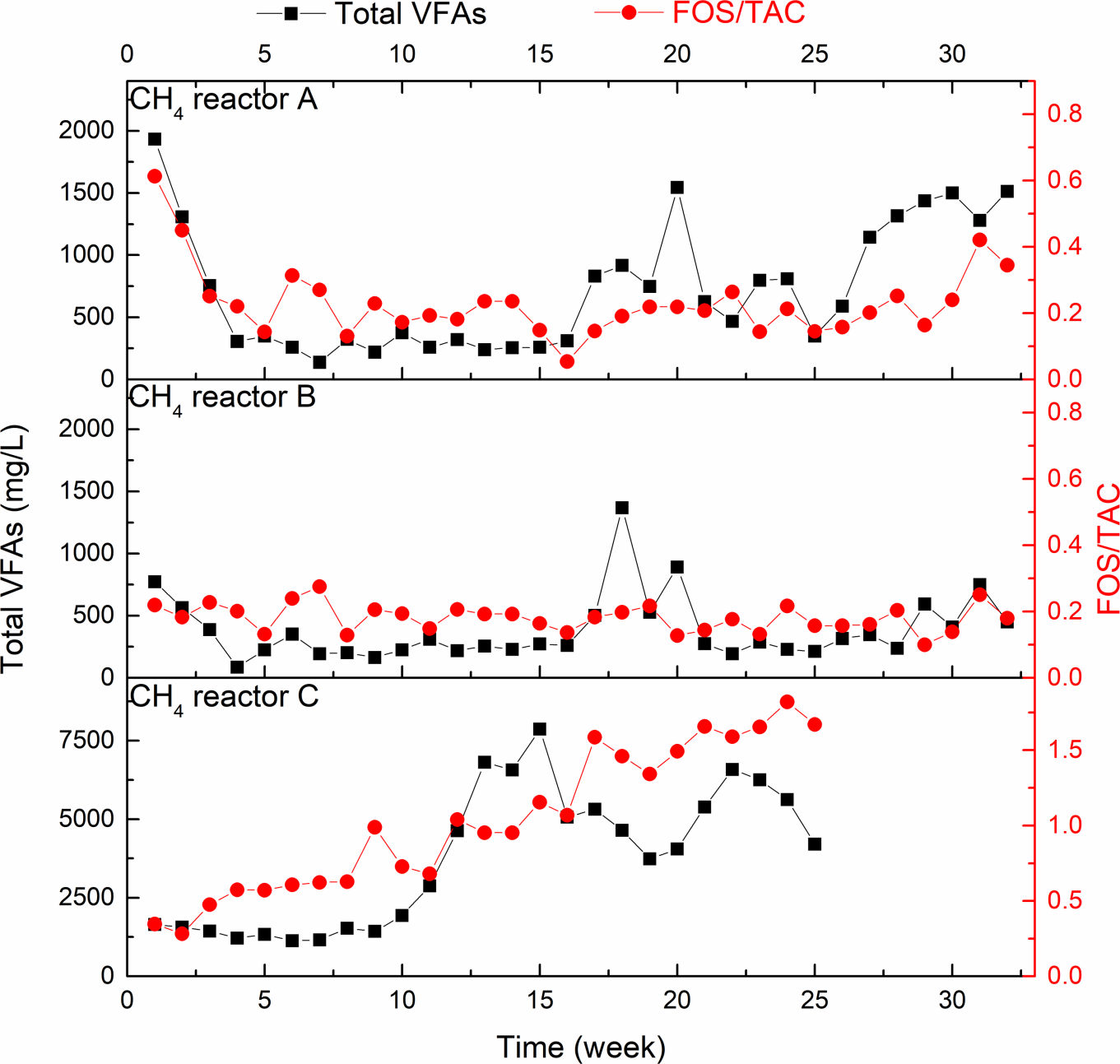

H2 reactor (HRT=4 d)
CH4 reactor A (HRT=12 d)
CH4 reactor B (HRT=24 d)
CH4 reactor C (HRT=16 d)
Specific hydrogen yield (mL/gVS)
Specific methane yield (mL/gVS)
Organic loading rate (gVS/L/d)
Time (week)


(a)
Heptanoic
Caproic
Iso caproic
Hydrogen
Valeric
Iso valeric
Butyric
Iso butyric
Propionic
Acetic
Volatile fatty acid (mg/L)
Specific hydrogen yield (mL/gVS)
Organic loading rate (gVS/L/d)
0
1000
2000
3000
4000
5000
6000
7000
8000
0
20
40
60
3
6
9
12


(b)
Heptanoic
Caproic
Iso caproic
Valeric
Iso valeric
Butyric
Iso butyric
Propionic
Acetic
Methane
Volatile fatty acid (mg/L)
Specific methane yield (mL/gVS)
Organic loading rate (g/L)
0
300
600
900
1200
1500
0
50
100
150
200
250
300
1
2
3
4


(c)
Heptanoic
Caproic
Iso caproic
Methane
Valeric
Iso valeric
Butyric
Iso butyric
Propionic
Acetic
1500
1200
900
600
300
0
300
250
200
150
100
50
0
Volatile fatty acid (mg/L)
Specific methane yield (mL/gVS)
0.5
1.0
1.5
2.0
Organic loading rate (gVS/L/d)


(d)
Heptanoic
Caproic
Iso caproic
Methane
Valeric
Iso valeric
Butyric
Iso butyric
Propionic
Acetic
Volatile fatty acid (mg/L)
Specific methane yield (mL/gVS)
Organic loading rate (gVS/L/d)
0
1000
2000
3000
4000
5000
6000
7000
8000
0
50
100
150
200
250
300
1
2
3
4


Total VFAs
FOS/TAC
CH4 reactor A
CH4 reactor B
CH4 reactor C
Total VFAs (mg/L)
FOS/TAC
Time (week)